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Identification and further characterization of selected udder pathogens isolated from bovine mastitis milk

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vorgelegt von

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Contents

1 Abstract

Within this thesis, strain characteristics and antibiotic resistance profiles of the two main udder pathogens *S. aureus* and *E. coli* are provided and a new PCR based identification system for *A. pluranimalium* is described.

A total of 78 *S. aureus* strains were further characterized. In the Staphaurex test, 49% of the isolates were latex-positive and 51% latex-negative. All latex-negative strains were assigned to CC151, whereas latex-positive strains were assigned to various clonal complexes. While the latex-negative isolates were susceptible to all antimicrobial agents tested, 13 % of latex-positive isolates were resistant to both ampicillin and penicillin. Microarray profiles of latex-negative isolates were highly similar, but differed largely from those of latex-positive isolates. Our findings suggest latex-negative isolates to represent a group of closely related strains with specific resistance and virulence gene patterns.

Eighty-three *E. coli* strains were tested for their sensitivity to various antibiotics and genotyped by pulsed field gel electrophoresis (PFGE). 16.9 % of the isolates were resistant to one or more antimicrobial agents and 10.8 % were multiresistant. Amoxicillin-clavulanic acid, gentamicin and third generation cephalosporins proved effective against the majority of these strains. Nevertheless, one $bla_{\text{CTX-M-14}}$ harbouring ESLB-producing strain was found. Genotyping demonstrated that *E. coli* from cows with mastitis were very diverse.

To identify *A. pluranimalium*, primers (PlaF/PlaR) based on the pluranimaliumlysin (*pla*) gene were designed and evaluated. The primer pairs revealed a correctly sized amplification product (458 bp) with all target strains tested, whereas no amplification product was obtained for all non-target strains.

Key words: bovine mastitis, antibiotic resistance, genotyping, main udder pathogens, *A. pluranimalium*

Zusammenfassung

In dieser Arbeit werden Stamm-Eigenschaften der zwei häufigsten Mastitis-Erreger *S. aureus* und *E. coli* besprochen. Zudem wird ein neues PCR basiertes Identifikationssystem für *A. pluranimalium* beschrieben.

78 *S. aureus*-Stämme wurden weitergehend charakterisiert. Im Staphaurex Test waren 51% aller Isolate negativ. Alle Test-negativen Stämme gehörten zum CC151 während die Test-positiven Stämme verschiedenen CC zugeordnet wurden. Die Test-negativen Isolate waren empfindlich gegen alle getesteten Antibiotika, jedoch zeigten 13% der Test-positiven Isolate Resistenz gegen Ampicillin und Penizillin. Die Microarray-Profile der Test-negativen Isolate waren sich sehr ähnlich aber unterschieden sich deutlich von denen der Test-positiven.

83 *E. coli*-Stämme wurden auf ihre Empfindlichkeit gegen verschiedene Antibiotika getestet und mittels PFGE genotypisiert. 16.9% aller Isolate waren resistent gegen eines oder mehrere Antibiotika und 10.8% waren multiresistent. Amoxicillin-Clavulansäure, Gentamicin und Cephalosporine der 3. Generation erwiesen sich als wirksam gegen die Mehrheit der Stämme. Die Genotypisierung zeigte, dass *E. coli*-Stämme isoliert von Kühen mit Mastitis sehr unterschiedlich sind.

Zur Identifizierung von *A. pluranimalium* wurden Primer basierend auf dem Pluranimaliumlysin-Gen designt und evaluiert. Das Primer-Paar führte bei allen Zielstämmen zu einem Amplikon der richtigen Größe (458bp) während bei den Nicht-Zielstämmen kein Amplikon generiert wurde.

Schlüsselwörter: bovine Mastitis, Antibiotikaresistenzen, Genotypisierung, häufigste Mastitiserreger, *A. pluranimalium*

2 Comparison of genomic and antimicrobial resistance features of Staphaurex latex test positive and Staphaurex latex test negative *Staphylococcus aureus* causing bovine mastitis

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2.1 Interpretive Summary

More than 50% of *Staphylococcus* (*S.*) *aureus* strains causing bovine mastitis are latex test negative in the Staphaureux latex agglutination test. Our study associated the latex agglutination phenotype of 78 *S. aureus* bovine mastitis isolates from 57 Swiss farms with genomic and antibiotic resistance features. We identified major differences between latex test positive and latex test negative strains with regard to antibiotic resistance, virulence gene profiles, and the assigned clonal complexes. The generated data provides new insights into genomic features of latex test positive and latex test negative strains. It also contributes to the identification of potential vaccine targets.

2.2 Abstract

The dairy industry suffers from massive economic losses due to staphylococcal mastitis in cattle. Staphaureux latex agglutination test was reported to lead to negative results in 54% of bovine Staphylococcus aureus strains and latex-negative strains were hypothesized to be less virulent than Staphaurex test positive strains. However, comparative information on virulence and resistance profiles of these two groups of *S. aureus* is scarce. Our objective was to associate the latex agglutination phenotype of *S. aureus* strains isolated from bovine mastitis milk with data on clonal complexes, virulence genes, and antibiotic resistance in order to 1) determine the virulence profiles of the Staphaureux test positive and Staphaurex test negative groups, and 2) provide data needed to improve treatment of bovine mastitis and to identify potential vaccine targets. A total of 78 *S. aureus* strains isolated from 78 different cows at 57 Swiss farms were characterized. Latex agglutination was tested by Staphaureux kit and resistance profiles were generated by disk diffusion. DNA microarray was used to assign clonal complexes and to determine virulence and resistance gene profiles. In the Staphaureux test, 49% of the isolates were latex-positive and 51% latex-negative. All latex-negative strains were assigned to CC151, whereas latex-positive strains were assigned to various clonal complexes including

CC97 (n = 16), CC8 (n = 10), CC479 (n = 5), CC20 (n = 4), CC7 (n = 1), CC9 (n = 1) and CC45 (n = 1). While the latex-negative isolates were susceptible to all antimicrobial agents tested, 24% of latex-positive isolates were classified intermediate with regard to cefalexin-kanamycin and 13 % were resistant to both ampicillin and penicillin. Microarray profiles of latex-negative isolates were highly similar, but differed largely from those of latex-positive isolates. While the latex-negative group lacked several enterotoxin genes and *sak*, it exhibited significantly higher prevalence rates of genes encoding enterotoxin C, toxic shock syndrome toxin, and leukocidins (*lukM/lukF-P83, lukD*). Our findings suggest latex negative isolates to represent a group of closely related strains with specific resistance and virulence gene patterns.

Key words: *Staphylococcus aureus*, bovine mastitis, latex agglutination, virulence

2.3 Introduction

The dairy industry suffers from massive economic losses due to staphylococcal mastitis in cattle (Wells et al., 1998). The Staphaurex latex agglutination test represents a diagnostic instrument widely used to confirm putative *Staphylococcus* (*S.*) *aureus* isolates through detection of characteristic *S. aureus* surface proteins. Latex particles coated with human IgG and fibrinogen interact with the bacterial target proteins *SpA* (staphylococcal protein A), *ClfA/B* (clumping factor A/B), and *FnbA/B* (fibronectin-binding protein A/B), mediating a rapid agglutination reaction visible to the naked eye. While the Staphaurex latex agglutination test exhibits high specificity (99.5%) and sensitivity (99.8%) when applied to *S. aureus* strains obtained from humans, Stutz et al. (Stutz et al., 2011) reported that 54% of *S. aureus* isolates obtained from cases of bovine mastitis yield negative test results. These false-negative results are due to sequence polymorphisms leading to impaired functionality of one or several of the targeted virulence factors *SpA*, *ClfA/B*, and *FnbA/B*. Therefore, Staphaurex latex agglutination test negative (SLAT(-)) strains have been

hypothesized to be less virulent than Staphaurex latex agglutination test positive (SLAT(+)) strains (Stutz et al., 2011). Although the assessment of the virulence potential of SLAT(-) strains is of crucial importance to the dairy industry, data on the genomic background and antimicrobial resistance of bovine SLAT(-) isolates is scarce.

Though antibiotic treatment is widely used to fight bovine mastitis, its merits are controversially discussed. Use of antimicrobial agents is not only economically questionable and favors the development of antibiotic resistance, but it is also unsuitable to address the issue of intracellular persistance of the organism (Fluit, 2012; Saini et al., 2012; Steeneveld et al., 2011). Therefore, increased efforts are now focused on the development of vaccines. Recent studies postulate extended characterization of the genetic background of bovine mastitis isolates to enable identification of proteins crucial for colonization and infection that could serve as biomarkers in the identification of vaccine targets (Fluit, 2012; Klein et al., 2012).

The objective of this study is to link the latex agglutination phenotype of *S. aureus* strains isolated from bovine mastitis milk with data on clonal complexes, virulence genes and antibiotic resistance in order to 1) determine the virulence profiles of the SLAT(+) and SLAT(-) groups, and 2) provide data needed to improve treatment of bovine mastitis and to identify potential vaccine targets.

2.4 Materials and Methods

2.4.1 Bacterial isolates, DNA extraction and presumptive species identification

A total of 78 *S. aureus* strains was isolated from bovine mastitis milk samples collected from different cows at 57 Swiss farms between March 2011 and Feb 2012. Putative *S. aureus* isolates were identified by streaking samples onto rabbit plasma fibrinogen plates (Oxoid, Basel, Switzerland), which were subsequently incubated at 37°C and examined for coagulase activity after 48 h. A single *S. aureus* typical colony of each plate was transferred to blood agar and incubated over night at 37°C. DNA isolation kits were supplied by QIAGEN (Hilden, Germany) and handled

according to the manufacturer's instructions. The concentration of nucleic acids was measured using Nanodrop ND-1000 UV/Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE).

2.4.2 Staphaurex Latex Agglutination Test

Staphaurex test kit (Oxoid, Basel, Switzerland) was used according to the manufacturer's instructions to determine latex agglutination.

2.4.3 Genotyping using DNA microarray

Presence of 284 genes and allelic variants was assessed using StaphyType ArrayStrips (Clondiag chip technologies, Jena, Germany) following the manufacturer's instructions. Multiplex linear DNA amplification and microarray hybridization allowed for identification of markers, genes conferring resistance to antimicrobial agents, and virulence determinants such as genes encoding enterotoxins, toxic shock syndrome toxin, leukocidins, hemolysins, and adhesins. The microarray also enables assignment of strains to clonal complexes and *agr* types. DNA microarray profiles were converted to sequence-like strings as described elsewhere to allow for visualization by SplitsTree4, a software package designed to compute unrooted phylogenetic networks from molecular sequence data (Huson et al., 2006; Wattinger et al., 2012).

2.4.4 Susceptibility testing

Disk diffusion was used to classify isolates as susceptible, intermediate or resistant depending on respective zone diameters following CLSI standard protocols (Clinical and Laboratory Standards Institute, 2008). All antimicrobial agents were chosen with regard to their relevance in mastitis therapy. Antibiotic agents tested included

ampicillin (30 µg), amoxicillin (20 µg) with clavulanic acid (10 µg), cephalothin (30 µg), ceftiofur (30 µg), erythromycin (15 µg), cefoxitin (30 µg), gentamicin 10 (µg), kanamycin (30 µg), kanamycin-cefalexin (30 µg/ 15 µg), penicillin (10 U.I.), and penicillin-novobiocin (10 U.I/ 30 µg). Mueller-Hinton agar, as well as disks containing ceftiofur and penicillin-novobiocin were provided by Oxoid, while disks containing cefalexin-kanamycin (Ubrolexin®) were provided by Boehringer Ingelheim (Basel, Switzerland). All other disks containing antibiotic agents were obtained from Becton Dickinson (Basel, Switzerland). Reference strain *S. aureus* ATCC 25923 was used as a quality control.

2.4.5 Statistical analysis

The distribution of specific genes among latex-positive and latex-negative isolates was compared based on the hybridization results of the DNA microarray. SPSS Statistics 19 (SPSS Inc., Chicago, IL) was used to run Pearson's chi-squared test, identifying significant associations between the latex-phenotype and the presence of the examined genes. Results were considered to be statistically significant for *p*-values < 0.050.

2.5 Results

2.5.1 Species confirmation and exclusion criteria

All isolates were confirmed to represent *S. aureus* using the species markers of the DNA microarray. To avoid bias, the sample collection was screened for identical isolates by comparison of all features tested including microarray profiles and resistance patterns, and all isolates were found to be unique.

2.5.2 Latex agglutination and clonal complexes

Latex agglutination was tested for all 78 confirmed bovine *S. aureus* strains. While 38 isolates (49%) were latex-positive and would have been correctly identified as *S. aureus*, 40 isolates (51%) were latex-negative (false-negative). As depicted in the Splitstree in Figure 1, all latex-negative strains exhibit very similar resistance and virulence gene profiles. They form a single cluster of isolates assigned to CC151, while latex-positive strains were assigned to various clonal complexes including CC97 (n = 16), CC8 (n = 10), CC479 (n = 5), CC20 (n = 4), CC7 (n = 1), CC9 (n = 1), and CC45 (n = 1).

2.5.3 Resistance phenotypes (disk diffusion)

Resistance phenotypes determined by disk diffusion are listed in Table 1. The SLAT(-) isolates were susceptible to all antimicrobial agents tested. Among the SLAT(+) group, 24% of the isolates were classified intermediate with regard to cefalexin-kanamycin and 13% of isolates were classified resistant to both ampicillin and penicillin. No MRSA (methicillin resistant *S. aureus*) isolates were detected among the bovine mastitis strains investigated in this study.

2.5.4 Resistance and virulence gene profiles

Selected DNA microarray results on resistance and virulence genes are depicted in Table 2. While no resistance genes were detected among SLAT(-) isolates, some SLAT(+) isolates exhibited genes involved in resistance to antibiotic agents including *blaI/R/Z* (26%), *ermC* (3%), *fosB* (39%), and *vanB* (3%). Several SLAT(+) strains also displayed enterotoxin genes *entA* (21%), *entD* (26%), and *entJ* (16%), as well as *sak* encoding staphylokinase (26%), virulence factors that were not found among SLAT(-) isolates. In contrast, the SLAT(-) group exhibited significantly higher prevalence rates of both tested allelic variants of *entC*, as well as the *egc* enterotoxin gene cluster, genes encoding toxic shock syndrome toxin, and leukocidins *lukM/lukF-*

P83 and *lukD*. Neither *pvl*, encoding panton-valentine leukocidin, nor *etA/B/C*, encoding exfoliative toxins, were detected in this study. Microarray data on the presence of selected adhesin genes coding for target proteins of the Staphaureux test is presented in Table 2. While almost all strains were positive for one or more allelic variants of the adhesin genes tested, latex-positive and latex-negative isolates differ largely in the allelic variants found. A comprehensive overview of the prevalence rates of all genes tested is provided as a supplemental file that also comprises *p*-values used to determine significant differences in the prevalence of respective genes among SLAT(+) and SLAT(-) isolates.

2.6 Discussion

While Staphaureux test kit exhibits high specificity (99.5%) and sensitivity (99.8%) when applied to *S. aureus* strains obtained from humans, it frequently leads to false-negative results when applied to *S. aureus* isolates obtained from bovine mastitis milk. We detected 51% SLAT(-) isolates, consistent with the rate of 54% reported by Stutz et al. (Stutz et al., 2011). In both studies SLAT(+) isolates were associated with a wide range of clonal complexes, whereas the SLAT(-) isolates were assigned to CC151 only. CC151 was reported to represent the most prevalent clonal complex among *S. aureus* isolated from bovine milk obtained from both seemingly healthy, as well as clinically infected udders (Sakwinska et al., 2011; Schlotter et al., 2012; Stutz et al., 2011). The most frequent clonal complexes among SLAT(+) strains in our study included CC97, CC8, CC497, and CC20, while CC7, CC9, and CC45 were assigned to only one isolate each. Interestingly, no isolates were assigned to CC133, a dominant clonal complex among *S. aureus* isolated from the milk of seemingly healthy cows in Germany (Schlotter et al., 2012).

When antimicrobial susceptibility was determined, the resistance proportions among clonal complexes differed, with CC151, CC479, and CC20 representing the only clonal complexes that comprised no resistant isolates. These findings are consistent with an extensive study on antimicrobial resistance among Swiss and French bovine *S. aureus* isolates, in which penicillin resistance rates among CC151 and CC20 were

found to be far lower than those among CC97 (Sakwinska et al., 2011). Overall, a total of 6% of bovine *S. aureus* isolates tested in our study were resistant to both ampicillin and penicillin, and 12% exhibited intermediate susceptibility to kanamycin/cefalexin. While the emergence of livestock-associated MRSA represents an increasing problem in Switzerland (Huber et al., 2010) and worldwide, no MRSA isolates were detected among the mastitis isolates investigated in our study. The single strain exhibiting *vanB*, one of the genes involved in vancomycin resistance, was found to be sensitive to vancomycin by Etest.

DNA microarray results indicate that the resistance and virulence gene profiles of the SLAT(-) strains in our study are highly similar, but differ largely from those of SLAT(+) strains (see Splitstree, Figure 1). Epidemiological studies suggest that a subset of *S. aureus* strains exhibits a distinctive genetic background that renders them highly successful in causing bovine mastitis (Herron-Olson et al., 2007). The SLAT(-) isolates characterized in our study lack several virulence genes frequently found among SLAT(+) isolates, including genes coding for resistance factors, staphylokinase, and enterotoxins A, D, and J. This particular combination of enterotoxin genes *entA*, *entD*, and *entJ* was recently described to represent one of the main criteria in the identification of *S. aureus* strains classified as genotype B (Boss et al., 2011). Strains of this genotype were reported to be exclusively associated with very high (up to 65%) within-herd prevalence of mastitis (Graber et al., 2009). In our study, strains exhibiting the combination of *entA*, *entD*, and *entJ* were assigned to CC8 only. A wide variety of enterotoxin genes can be found among *S. aureus* isolates obtained from cases of bovine mastitis (Monecke et al., 2007). Still, the exact role of enterotoxins in the pathogenesis of bovine mastitis remains poorly understood (Haveri et al., 2007).

Among SLAT(-) strains, we found significantly higher numbers of several other virulence genes, including genes coding for toxic shock syndrome toxins (*tst-1, tst-RF122*), enterotoxins (*entC, egc*-cluster), and leukocidins (*lukM/lukF-P83, lukD*). Recent studies suggest *lukM/lukF-P83* to play a role in the pathogenesis of bovine mastitis (Barrio et al., 2006; Schlotter et al., 2012).

As false-negative results in the Staphaurex test are due to impaired functionality of one or several of the targeted virulence factors *SpA*, *ClfA/B*, and *FnbA/B*, SLAT(-) strains have been hypothesized to be less virulent than SLAT(+) strains (Stutz et al., 2011). The DNA microarray results revealed considerable heterogeneity regarding

clfA/B and *FnbA/B*, and we found SLAT(-) and SLAT(+) isolates to exhibit different alleles of the respective genes. *ClfA/B* and *FnbA/B* are representatives of the group of bacterial surface proteins designated MSCRAMMSs (microbial surface components recognizing adhesive matrix molecules). These proteins are of particular interest in the development of vaccines, as they mediate adherence of *S. aureus* to components of the host's extracellular matrix. Attachment of the organism to the epithelial cells of the udder, as well as subsequent invasion of epithelial and endothelial cells was shown to depend on MSCRAMMs (Lammers et al., 1999; Sinha et al., 1999), which also play a role in the evasion of host immune response (Higgins et al., 2006).

2.7 Conclusions

The genomic background of SLAT(-) and SLAT(+) strains differs significantly, including a vast range of genes encoding crucial virulence and resistance factors. In consideration of the high heterogenity among MSCRAMM genes detected by DNA microarray, we conclude that a combination of diverse antigens is crucial to the development of highly functional adhesin-based diagnostic tools and vaccines. Based on the comparison of microarray profiles and resistance phenotypes of SLAT(+) and SLAT(-) bovine mastitis isolates investigated in this study, we hypothesize that SLAT(+) strains exceed SLAT(-) strains in virulence potential.

2.8 Acknowledgements

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2.10 Tables

Table 1: Antimicrobial resistance phenotypes determined by disk diffusion among Staphaurex latex agglutination test positive (SLAT(+)) and Staphaurex latex agglutination test negative (SLAT(-)) isolates

	Antimicrobial agents[1]										
	AM	AMC	CF	EFT	ERY	FOX	GM	K	K/CFX	P	P/NB
SLAT(-)											
CC151 (n = 40)	0	0	0	0	0	0	0	0	0	0	0
SLAT(+)											
CC7 (n = 1)	1	0	0	0	0	0	0	0	0	1	0
CC8 (n = 10)	1	0	0	0	0	0	0	0	(1)[2]	1	0
CC9 (n = 1)	1	0	0	0	0	0	0	0	(1)[2]	1	0
CC20 (n = 4)	0	0	0	0	0	0	0	0	(3)[2]	0	0
CC45 (n = 1)	1	0	0	0	0	0	0	0	0	1	0
CC97 (n = 16)	1	0	0	0	0	0	0	0	(4)[2]	1	0
CC479 (n = 5)	0	0	0	0	0	0	0	0	0	0	0
Total (n = 78)	5	0	0	0	0	0	0	0	(9)[2]	5	0

[1]) Abbreviations: AM = ampicillin, AMC = amoxicillin with clavulanic acid, CF = cephalothin, EFT = ceftiofur, ERY = erythromycin, FOX = cefoxitin, GM = gentamicin, K = kanamycin, K/CFX = kanamycin-cefalexin, P = penicillin, P/NB = penicillin-novobiocin

[2]) The number of isolates that exhibited intermediate sensitivity to the respective antimicrobial agent is presented in brackets.

Table 2: Prevalence of selected virulence and resistance genes detected by DNA microarray among Staphaurex latex agglutination test (SLAT(+)) and Staphaurex latex agglutination test negative (SLAT(-)) bovine mastitis isolates

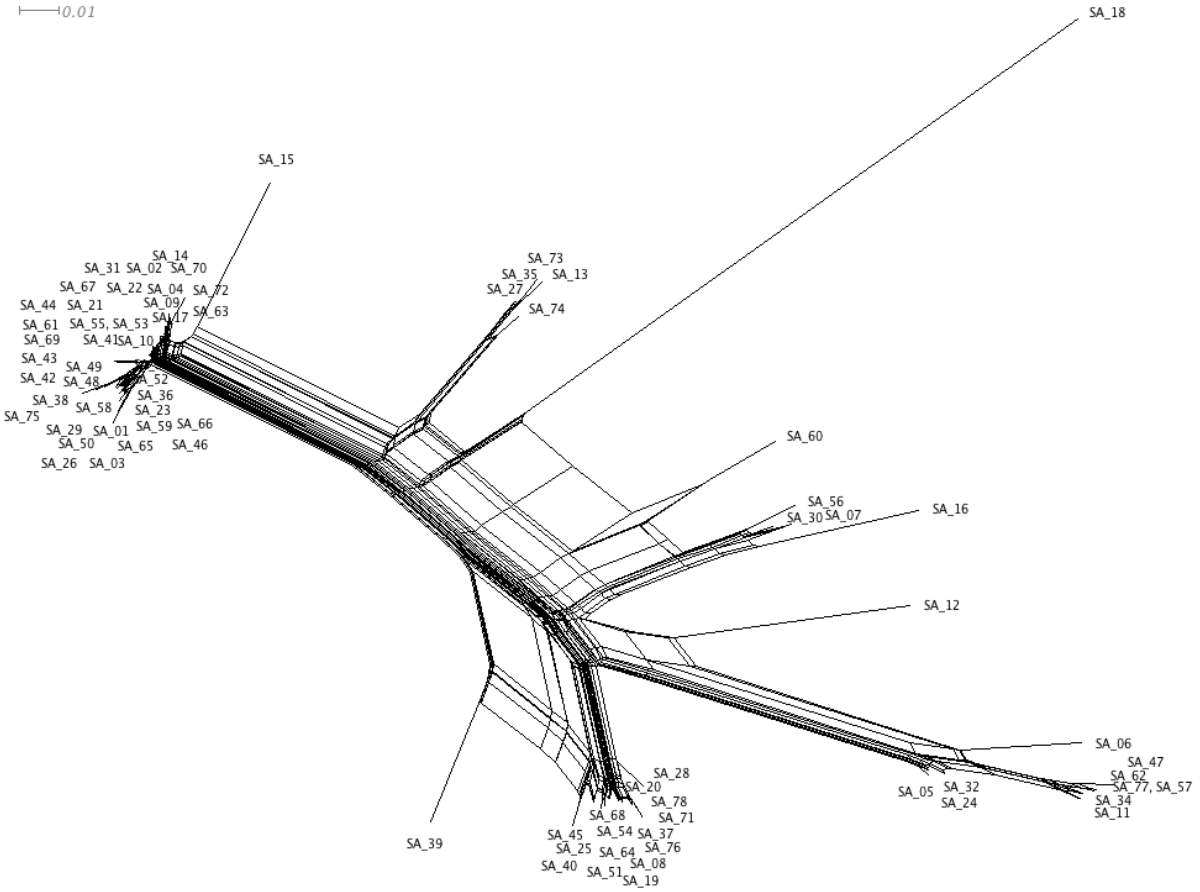
Group	Gene/Probe	Function	SLAT(+) (n = 38)	SLAT(-) (n = 40)
agr typing	agrI	accessory gene regulator, type I	84%	0%*
	agrII	accessory gene regulator, type II	16%	100%*
Capsule	capsule-5	capsule type 5	79%	0%*
	capsule-8	capsule type 8	21%	100%*
Resistance	blaI, blaR, blaZ	beta lactamase resistance	26%	0%*
	fosB	putative marker for fosfomycin, bleomycin	39%	0%*
Enterotoxins	entA	enterotoxin A	21%	0%*
	entC	enterotoxin C	3%	28%*
	entCM14	enterotoxin-like protein	3%	100%*
	entD	enterotoxin D	26%	0%*
	entJ	enterotoxin J	16%	0%*
	egc-cluster	enterotoxin gene cluster	29%	100%*
Toxic shock	tst-1	toxic shock syndrome toxin	0%	13%*
	tst-RF122	allelic variant of toxic shock syndrome toxin from RF122	0%	15%*
Leukocidins	lukM/lukF-P83	bovine leukocidin	42%	100%*
	lukD	leukocidin D component	87%	100%*
Staphylokinase	sak	staphylokinase	26%	0%*
Spa	spa	staphylococcal protein A	100%	100%
ClfA/B	clfA-all	clumping factor A (ClfA)	97.4%	100%
	clfA-COL+RF122	ClfA, allele from COL/ RF122	71%	100%*
	clfB-COL+Mu50	ClfB, allele from COL/ Mu50	26%	0%*
	clfB-MW2	ClfB, allele from MW2	5%	100%*
	clfB-RF122	ClfB, allele from RF122	18%	100%*
FnbA/B	fnbA	fibronectin-binding protein A (FnbA)	89%	100%*

	fnbA-COL	FnbA, allele from COL	29%	0%*
	fnbA-MRSA252	FnbA, allele from	16%	0%*
	fnbA-RF122	FnbA, allele from RF122	0%	100%*
	fnbB-COL+ Mu50+MW2	fibronectin-binding protein B (FnbB)	100%	0%*

*The prevalence of the respective gene differs significantly between the SLAT(+) and SLAT(-) bovine S. aureus isolates tested in this study ($p < 0.050$).

2.11 Figures

Figure 1: Splitstree depicting similarity of resistance and virulence gene profiles among SLAT(+) and SLAT(-) bovine mastitis isolates. CC151 represents the only cluster comprised of SLAT(-) isolates. The latex phenotype of all isolates clustered in one clonal complex is indicated in brackets.



3 Resistance profiles and genetic diversity of *Escherichia coli* isolated from acute bovine mastitis

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3.1 Abstract

Between March 2011 and February 2012 83 *E. coli* strains were isolated from mastitis milk samples from 83 different animals (67 farms) and tested for their sensitivity to various antibiotics by means of disk diffusion method and genotyped by determination of the phylogenetic groups as well as by pulsed field gel electrophoresis (PFGE). The antibiotics were chosen on the basis of their licenses for intramammary application in Switzerland. As many as 16.9 % of the isolates were resistant to one or more antimicrobial agents. Amoxicillin-clavulanic acid, gentamicin and third generation cephalosporins proved effective against the majority of these strains. Nevertheless, one *bla*$_{CTX\text{-}M\text{-}14}$ harbouring extended-spectrum-beta-lactamase producing strain was found. Genetic analysis grouped most of the strains (87%) into phylogenetic groups A and B1. PFGE genotyping demonstrated that *E. coli* from cows with mastitis even from the same farm were genotypically very diverse.

Keywords: *E. coli*, mastitis, resistance, genotyping, phylogenetic group, PFGE

Resistenzprofile und genetische Vielfalt von *Escherichia coli* Stämmen isoliert aus akuten bovinen Mastitiden

Zwischen März 2011 und Februar 2012 wurden 83 *E. coli* Stämme von 83 verschiedenen Kühen aus 67 Betrieben gesammelt und auf ihre Empfindlichkeit gegenüber verschiedenen Antibiotika getestet. Die Antibiotika wurden aufgrund der Zulassung für eine intramammäre Applikation in der Schweiz ausgesucht und die Empfindlichkeitstestung mittels Agardiffusions-Methode durchgeführt. Zudem wurden alle Stämme hinsichtlich ihrer Zugehörigkeit zu den phylogenetischen Gruppen wie auch mittels Pulsfeldgelelektrophorese (PFGE) genotypisiert. 16.9% aller Stämme zeigten Resistenzen gegenüber einem oder mehreren Antibiotika. Amoxicillin-Clavulansäure, Gentamicin und Cephalosporine der dritten Generation erwiesen sind als wirksam gegen die Mehrheit der *E. coli* Stämme. Jedoch wurde ein extended-Spectrum-beta-Lactamase Bildner, welcher das *bla*$_{CTX\text{-}M\text{-}14}$-Gen trägt, gefunden. Die genetische Analyse gruppierte das Gros der Stämme (87%) in die phylogentischen

Guppen A und B1. Die weitere Genotypisierung mittels PFGE zeigte eine grosse Diversität unter den *E. coli* Stämmen, auch wenn diese vom selben Betrieb stammten.

Schlüsselwörter: *E. coli*, Mastitis, Resistenz, Genotypisierung, phylogenetische Gruppen, PFGE

3.2 Introduction

Mastitis remains a major challenge to the worldwide dairy industry. For Switzerland, the average annual cost due to clinical mastitis has been estimated to be about SF 300.- per cow (Rüsch, 1995). Escherichia coli is the most common Gram-negative bacterium causing acute mastitis in cows worldwide (Hogan and Smith, 2003, Ericsson Unnerstad et al., 2009). *E. coli* induced mastitis is characterized as a relatively short-term disease process and induces a distinct acute phase response.
Antimicrobial treatment of an acute clinical mastitis has to start before the results of antimicrobial susceptibility testing are available because of the very often peracute course of the disease. Knowledge on current resistance patterns guides this "empiric" treatment and will enable a more accurate use of antibiotics. The latest published data on resistance profiles of *E. coli* causing mastitis in cows in Switzerland are about 10 years old (Stephan and Rüsch, 1997; Corti et al., 2003). In the meantime extended spectrum β-lactamases (ESBL) producing *E. coli* isolated from milk of cows with clinical mastitis were described (Locatelli et al., 2009). And recently, a CMY-2 β-lactamase producing *E. coli* (plasmid-mediated AmpC-producing *E. coli*) isolated from a cow with recurrent mastitis, was found in Switzerland (Endimiani et al., 2012). Therefore, from a clinical perspective, current data about resistances profiles of *E. coli* are required.
The *E. coli* species encompasses both pathogenic and non-pathogenic strains. Pathogenic strains cause a variety of enteric and extraintestinal infections in humans and animals, mostly in a host- or organ-specific way. Nevertheless, with regard to bovine mastitis, an *E. coli* pathogenic subset has not been identified yet. Bovine

mastitis *E. coli* do not belong to specific antigen O serogroups (Wenz et al., 2006) and are not biochemically different from fecal *E. coli* (Nemeth et al., 1994). Because of this apparent lack of specific features, it has been largely accepted that there is no strain specificity in *E. coli* bovine mastitis and that various *E. coli* strains found in the environment bear the same potential to cause the disease (Burvenich et al., 2003, Fernandes et al. 2011). The aim of this study was to retrieve current data on resistance profiles of *E. coli* strains form bovine mastitis milk samples as well as on the degree of genetic variability among isolates in Switzerland.

3.3 Material and Methods

3.3.1 Strains

A total of 83 *E. coli* strains from acute clinical cases of bovine mastitis were isolated from 83 different animals (67 different farms distributed in the cantons of Zurich, Graubünden, Thurgau, Aargau, St. Gallen, Uri and Zug). Not more than two cows per farm were included. The isolates were collected between March 2011 and February 2012. Milk samples were taken during farm calls by the attending veterinarian from the affected quarter of each cow in an aseptical manner.

Using a sterile loop, the samples were streaked onto sheep blood agar base (Beckton Dickinson AG, Allschwil, Switzerland), supplemented with 5 % sheep blood (Oxoid, Pratteln, Switzerland) as well as onto BROLACIN-Agar (VWR International AG, Dietikon, Switzerland) and incubated at 37 °C overnight. The *E. coli* strains were confirmed by colony morphology, Gram stain, and biochemical tests such as acid production from mannit, ONPG test, tests for urease, indol and H_2S production and the lysindecarboxylase test. The strains were stored at -80°C.

3.3.2 Susceptibility testing

The strains were subjected to antimicrobial susceptibility testing using the standard disk diffusion test according to the protocols recommended by the Clinical and Laboratory Standards Institute (2008). The antimicrobial agents tested as well as the corresponding interpretative criteria are summarized in Table 1. Bacteria were grown 4 – 5 h in BHI (Brain Heart Infusion, Oxoid, Pratteln, Switzerland) and diluted to 0.5 Mac Farland turbidity by comparison with a standard. Thereafter, bacterial suspensions were swabbed uniformly across a Müller-Hinton-Agar (Oxoid, Pratteln, Switzerland) plate and the filter-paper disks impregnated with the antimicrobial were placed on the surface of the agar using a dispenser. The disks were provided from Beckton Dickinson AG, Allschwil, Switzerland, except Ceftiofur, which was provided from Oxoid, Pratteln, Switzerland. Plates were incubated at 35 °C for 18 h and the zone of inhibition (in mm) was assessed and measured using a calliper. Interpretive criteria according to the performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals (Clinical and Laboratory Standards Institute, 2008) were used. Accuracy of the test system was monitored by including the reference strain *E. coli* ATCC 25922.
Additionally the strains were cultured on Brilliance ESBL agar (Oxoid, Pratteln, Switzerland) and incubated for 24h at 37°C. Strains, which were able to produce blue colonies, were confirmed as ESBL producers on Muller-Hinton agar plates using E-Test-ESBL strips containing cefotaxime, cefepime or ceftazidime each alone and in combination with clavulanic acid (bioMérieux, Marcy l'Etoile, France) according to the manufacturer's recommendations.

3.3.3 Identification and sequencing of *bla*$_{ESBL}$ genes

Bacterial strains confirmed for producing ESBLs were further analysed by PCR and by sequencing the whole open reading frames (ORF) of *bla* genes. DNA was extracted by a standard heat lysis protocol. Thereafter, specific primer sets were used to search for b-lactamase-encoding genes belonging to *bla*$_{TEM}$, *bla*$_{SHV}$ and *bla*$_{CTX-M}$ families (Geser et al., 2011; Geser et al. 2012). Resulting amplicons were

purified using the PCR Purification Kit (QIAGEN, Courtaboeuf, France) according to the manufacturer's recommendations. Custom-sequencing was performed by Microsynth (Balgach, Switzerland) and the nucleotide and protein sequences were analyzed with Codon Code Aligner V. 3.7.1.1. For database searches the BLASTN program of NCBI (http://www.ncbi.nlm.nih.gov/blast/) was used.

3.3.4 Determination of the *E. coli* phylogenetic groups

Phylogenetic analyses have shown that *E. coli* strains fall into four main phylogenetic groups (A, B1, B2, D). All isolates in the collection were assigned to phylogenetic groups, in which group A/B1 typically contained commensal isolates and group B2/D isolates were associated with virulence, using a triplex PCR protocol (Clermont et al., 2000) after DNA extraction by a standard heat lysis protocol.

3.3.5 PFGE

Genetic variability of isolates was determined by macrorestricion analysis and pulsed field gel electrophoresis (PFGE) according to the PulseNet standardized protocol for the subtyping of *E. coli* O157*, Salmonella, Shigella* (Ribot et al., 2006). After electrophoresis the gels were ethidium bromide stained and the banding pattern was photographed under (UV) illumination and a digital image (that was converted to TIFF format) of the pattern was acquired using the GelDoc system (Bio-Rad). The TIFF images were analysed using the BioNumerics software GelCompare (Applied Maths, Sint-Martens-Latem, Belgium) and Dice coefficient and UPGMA was employed to generate dendrograms. Analysis parameters were set to 2 % for both optimization and tolerance values respectively.

3.4 Results and Discussion

3.4.1 Susceptibility testing

Prevalence data for resistances in view of the antibiotics tested are summarized in Table 1. In total, 16.9% (n=14) of the strains showed resistance to one or more antimicrobial agent: 2.4% (n=2) of all *E. coli* strains were resistant against gentamicin, 10.8% (n=9) were resistant against kanamycin, 15.7% (n=13) against ampicillin, 3.6% (n=3) against cephalothin and 1.2% (n=1) against ceftiofur. No resistant strains were found for the combination amoxicillin-clavulanic acid. Nine strains (10.8%) were resistant against more than one class of antibiotics, the most frequent ones showed resistance against kanamycin and ampicillin (7.2%, n=6). One strain (1.2%) showed resistance against kanamycin, ampicillin and cephalothin and another strain (1.2%) showed resistance against gentamicin, kanamycin and ampicillin. In addition we found one ESBL-producer, harbouring $bla_{\text{CTX-M-14}}$, which was also resistant against the aminoglycosides kanamycin and gentamicin. The distributions of the inhibition zones of all strains are shown in Figure 1.

A comparison of the results from this study with previous studies in Switzerland (Stephan and Rüsch, 1997, Corti et al., 2003) revealed no global changes in the resistance situation during the last 15 years. However, concerning cephalothin, 22 strains fell into the interpretative category "intermediate". This might indicate a shift of the population towards resistance and will need to be observed over the next years. By comparing the results of this study with recently published data from European countries (Hendriksen et al., 2008; Botrel et al., 2010) no obvious differences in view of resistance prevalence were evident.

3.4.2 Phylogenetic groups

Most of the isolates investigated could be assigned to phylogenetic groups A and B1, with 29/83 (34.9%) assigned to group A and 43/83 (51.8%) assigned to group B1. Eleven isolates (13.3%) were assigned to the group D and none to phylogenetic group B2. With regard to virulence, phylogenetic groups B2 and D are considered to

be more likely to carry pathogenicity-associated genes, while groups A and B1 are classified commensal strains (Clermont et al., 2000). In our study, most of the isolates belonged to groups A and B1. In this context, it is worth mentioning, that this was in agreement with findings from a survey showing that *E. coli* isolates, shed by healthy cattle predominantly belonged to lineages A and B1 (Houser et al., 2008). However, it cannot be excluded that such strains may harbour some factors which may favour e.g. colonization of the udder.

3.4.3 Genotyping

The genetic variability was high among the 83 *E. coli* mastitis isolates from the 67 farms, and most of the strains were clonally not related based on a cut off value of 95% (Fig. 2). Even isolates from multiple cows from the same farm displayed diverse PFGE patterns. Only three PFGE patterns (two strains each were indistinguishable) were found in different farms (Fig. 2). Our data confirm the results of Wenz et al. (2006), who also found a high heterogeneity among *E. coli* strains isolated from cows with mastitis. The high genetic heterogeneity among the strains underlines that, in contrast to *S. aureus* (Stutz et al., 2011), no specific *E. coli* clones are mainly responsible for the *E. coli* mastitis situation.

This fact further highlights, that besides the use of antibiotics, the strategy for treatment of mastitis should be flanked by preventive measures. These comprise a number of approaches such as maintenance of a clean, dry, comfortable environment; proper milking procedures; proper maintenance and use of milking equipment; appropriate management of clinical mastitis during lactation; effective dry cow management; maintenance of biosecurity for contagious pathogens and culling of chronically infected cows as well as regular monitoring of udder health status.

3.5 Acknowledgements

We would like to thank Helga Abgottspon for her technical assistance.

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3.7 Tables

Table 1: Antimicrobial agents used in this study, interpretative criteria[1] and prevalence of resistances (n=83)

Antimicrobial agents		resistant (inhibition zone diameters in mm)	susceptible (inhibition zone diameters in mm)	resistant strains (%)
Gentamicin (GM)	10µg	≤12	≥15	2.4
Kanamycin (K)	30µg	≤13	≥18	10.8
Ampicillin (AM)	10µg	≤13	≥17	15.7
Amoxicillin-clavulanic acid (AC)	20/10µg	≤13	≥18	0
Cephalothin (CF)	30µg	≤14	≥18	3.6
Ceftiofur (CFT)	30µg	≤17	≥21	1.2

[1]according to M31-A3: Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals; Approved Standard-Third Edition; CLSI, Wayne, USA 2008.

3.8 Figures

Figure 1: Distribution of inhibition zone diameters for the tested antimicrobial agents**.** (GM: gentamicin; K: kanamycin; AM: ampicillin; AC: amoxicillin-clavulanic acid; CF: cephalothin; CFT: ceftiofur)**.**

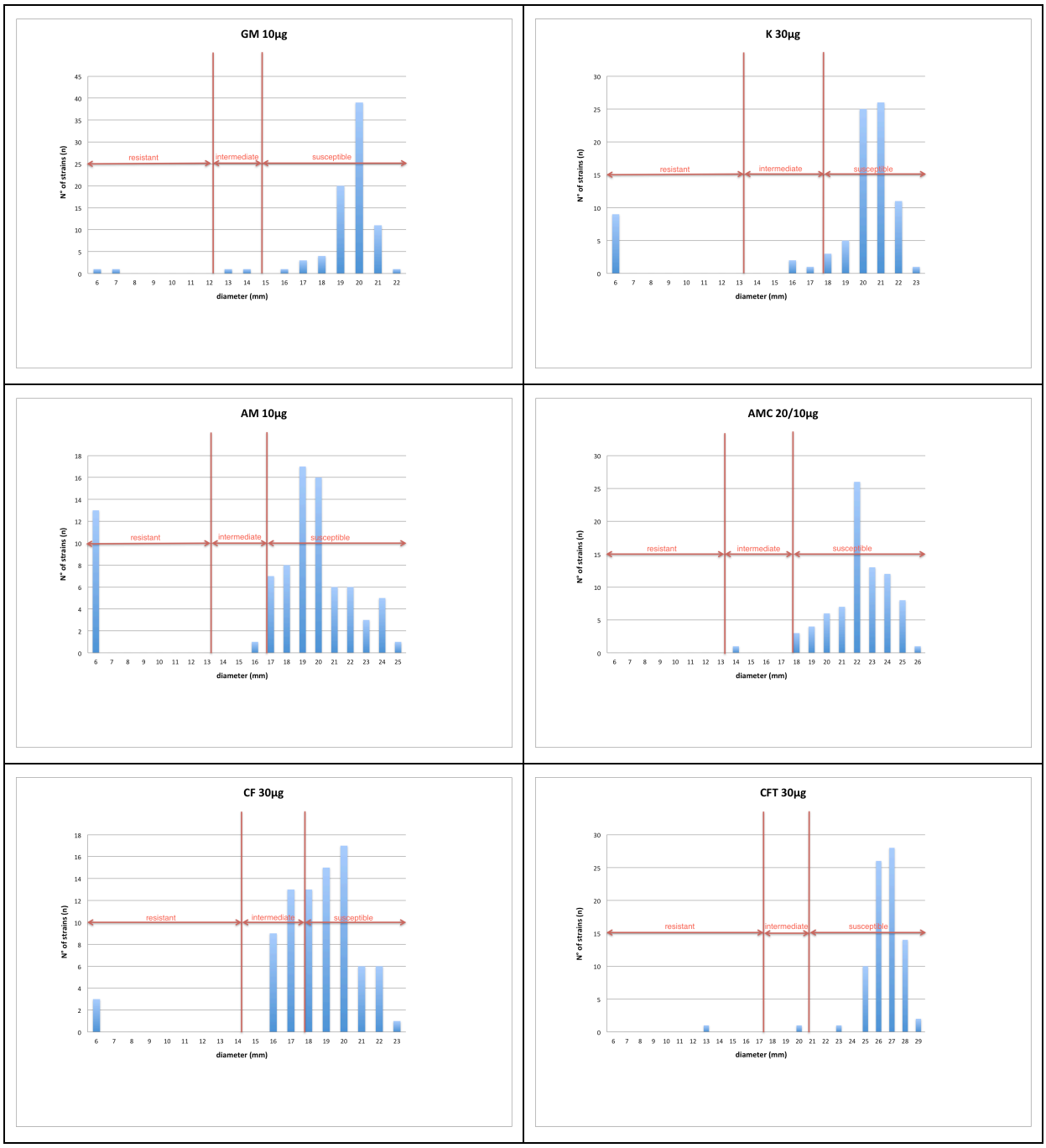
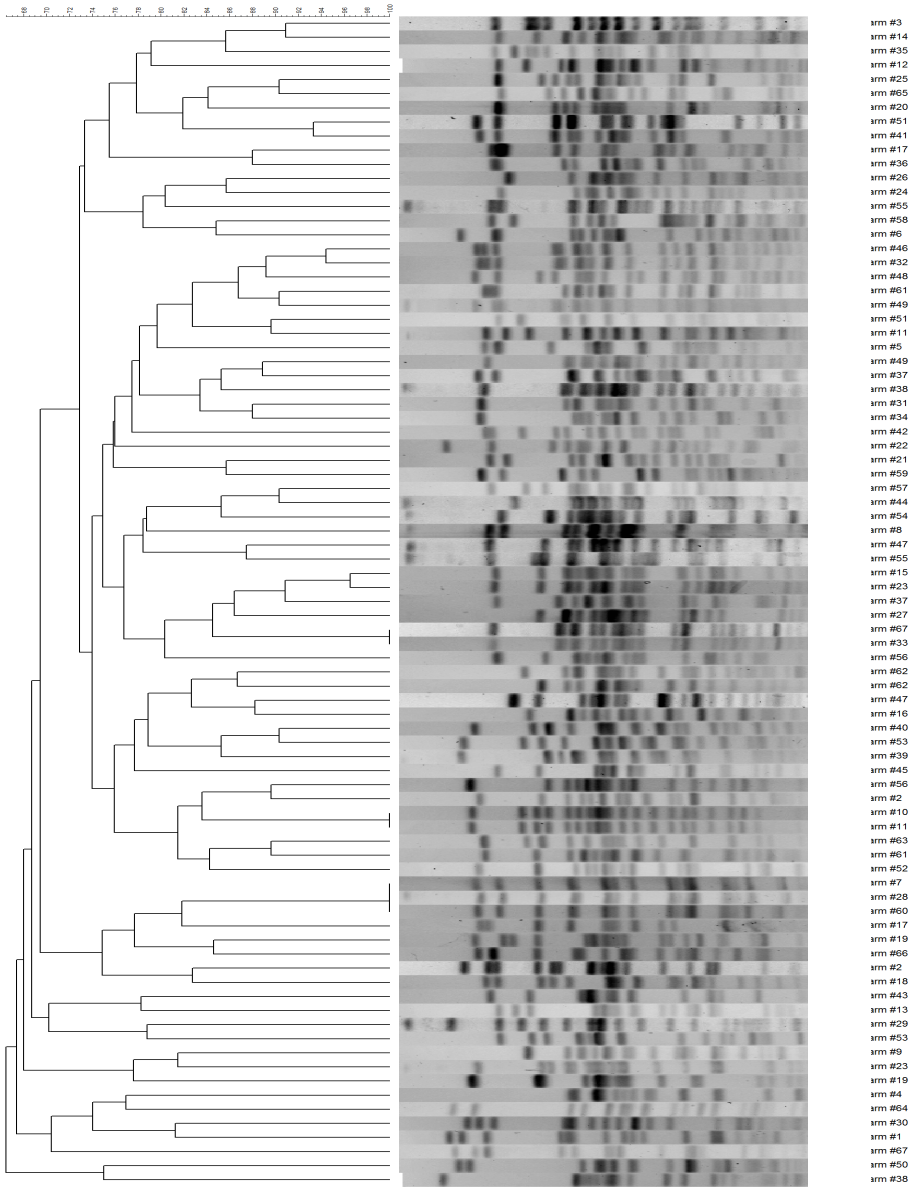


Figure 2: Cluster analysis and dendrogram of PFGE pattern of 83 *E. coli* isolates from mastitis milk samples. The dendrogram was generated using BioNumerics software GelCompare and Dice coefficient and UPGMA. Analysis parameters were set to 2 % for both optimization and tolerance values respectively. For clonal relationship, the cut off value (see line) was set at 95%.



4 *Arcanobacterium pluranimalium* leading to a bovine mastitis: Species identification by a newly developed *pla* gene based PCR

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4.1 Abstract

We are describing a clinical case of bovine mastitis due to *Arcanobacterium pluranimalium* in a Holstein-Friesian heifer, delivering bloody milk on the left hindquarter. Moreover, we report on the development and evaluation of PCR primers based on the pluranimaliumlysin (*pla*) gene for the identification of this species. With the primer pair PlaF/PlaR the *A. pluranimalium* type strain as well as the mastitis isolate 704 revealed a correctly sized amplification product (458 bp), whereas no amplification product was obtained for all non-target strains. The established PCR provides a new and convenient tool for the mastitis diagnostic to differentiate between *A. pluranimalium* and *Trueperella pyogenes*.

Keywords: *A. pluranimalium*, mastitis, PCR, identification

Bovine Mastitis verursacht durch *Arcanobacterium pluranimalium* und Etablierung einer Identifizierungs-PCR basierend auf dem *pla* Gen

Dieser Bericht beschreibt den Fall einer Holstein-Friesian Färse mit klinischer Mastitis (blutige Milch, Hinterviertel links), die durch *A. pluranimalium* verursacht wurde. Zudem wurden im Rahmen dieser Arbeit PCR Primer, basierend auf dem Pluranimaliumlysin (*pla*) Gen, zur Identifizierung von *A. pluranimalium* evaluiert. Sowohl der Referenzstamm wie auch das klinische Isolat 704 zeigten korrekte Amplifizierungsprodukte (458bp). Alle nicht-Zielstämme waren in der PCR negativ. Die etablierte PCR stellt damit für die Mastitis-Diagnostik ein praktisches Hilfsmittel dar, das zur Unterscheidung zwischen *A. pluranimalium* und *Trueperella pyogenes* herangezogen werden kann.

Schlüsselwörter: *A. pluranimalium*, Mastitis, PCR, Identifizierung

4.2 Case Report

4.2.1 Case history

In May 2012 the diagnostic department of the Institute of Food Safety and Hygiene, University of Zurich, received a milk sample from bovine origin. The cow, a Holstein-Friesian heifer, delivered bloody milk on the left hindquarter since beginning of May 2012 and decreased milk yield was observed. First, the owner assumed that another cow probably hit the cow. He also noticed that this quarter was harder than the other three ones. There was no injury, redness or swelling visible. The cow was in a good general condition. After 2 weeks without any change in milk appearance, a milk sample was aseptically taken and provided for bacteriological analysis. At this time the California Mastitis Test (CMT) from this quarter showed a score of 3 (Schalm et al., 1971).

4.2.2 Diagnostics

The milk was cultured on blood agar and incubated over night at 37°C. The next morning there was no visible growth on the plate. After additional 24 hours of incubation small (0.5 to 1mm), round, translucent to grey colonies producing a total hemolysis were visible (Fig. 1).

Such colonies showing a total hemolysis on blood agar are typical for *Trueperella pyogenes* (former *A. pyogenes;* Yassin et al., 2010). Nevertheless, Gram staining of these colonies showed no clear picture. MALDI-TOF based identification also yielded no result, since there was no match with the available spectra. It was therefore decided to further identify these colonies by molecular based methods. The isolate (named isolate 704) was investigated by 16S rDNA (primer pair: 616V, 1492R; Loy et al., 2002), 23 rDNA (primer pair: 23S-1, 23S-2; Ülbegi-Mohyla et al., 2010) and 16S-23S rDNA intergenic spacer region (primer pair: c and b; Kostman et al., 1995; Chanter et al., 1997; Hassan et al., 2008) sequencing using the amplification conditions described in the respective publications. Both, the 948 bp 16S rDNA amplicon (GenBank accession number JX144330) as well as the 663 bp 23S rDNA

PCR product (JX44331) yielded a 99% identity to the respective sequences of the corresponding *Arcanobacterium pluranimalium* reference strain M430/94/2 (DSM 13483^T). Intergenic spacer region PCR revealed a 534bp sized amplicon (JX144332) and a 98% identity to the corresponding *A. pluranimalium* reference strain sequence. The original description of *A. pluranimalium* is based on an isolate from a harbour poroise in Scotland and another from a fallow deer from Sweden (Lawson et al., 2001). In the years from 2001 to 2009, *A. pluranimalium* was recovered from 22 sheep samples as reported by Foster and Hunt (2011) including material from abortion, semen, abscesses, viscera and one case each of naval ill and peritonitis. Within this study a number of other host species were examined but there was only one further isolate of *A. pluranimalium* which was recovered from a milk sample collected from a cow with mastitis. Overall, this suggests that ovine animals are the major host of *A. pluranimalium,* with other host animals rarely affected.

4.2.3 Treatment and progress

After collecting a milk sample for the microbiological analysis, the left hindquarter of the cow was treated intramammarily with Neo-M (Dr. E. Graeub, Switzerland), which contain 1 Mio. U.I. Benzylpenicillinum procainum and 350mg Neomycinum per 10g. Four injectors were administered with an interval of 12 hours. A fifth injector was administered 24 hours after the fourth one. After the treatment the milk returned to normal character and milk yield increased again. Three weeks after milk sampling the quarter still showed a hardening but the CMT was negative.

4.3 Development and evaluation of a pluranimaliumlysin (*pla*) gene based *A. pluranimalium* specific identification PCR

Until recently the genus *Arcanobacterium* comprised nine species: *A. haemolyticum*, *A. pyogenes*, *A. bernardiae*, *A. phocae*, *A. bialowiezense*, *A. bonsai*, *A. hippocoleae*,

A. abortisuis and *A. pluranimalium*, many of which were associated with animal hosts and/or pyogenic and opportunistic infections (Collins et al., 1982, Lehnen et al., 2006). In 2010, comparative chemotaxonomic and phylogenetic studies performed by Yassin et al. (2010) indicated that the genus *Arcanobacterium* was not monophyletic and it was proposed that the genus *Arcanobacterium* should be restricted to *A. haemolyticum*, *A. hippocolae*, *A. phocae* and *A. pluranimalium*. The other species *A. abortisuis*, *A. bernardiae*, *A. bialowiezense*, *A. bonasi* and *A. pyogenes* were reclassified in a new genus *Trueperella*, as *Trueperella abortisuis*, *Trueperella bernardiae*, *Trueperella bialowiezensis*, *Trueperella bonasi* and *Trueperella pyogenes*.

So far, *T. pyogenes* and *A. pluranimalium* have been associated to bovine mastitis. Based on colony morphology and the hemolytic activity on blood agar, these two species cannot be differentiated from each other. Since no *A. pluranimalium* specific identification PCR had previously been available, a further aim of this study was to design and evaluate specific PCR primers for the identification of this species. A primer pair (PlaF: 5`- TCG CCA ATC AGA ATC TCG-3` and PlaR: 5`- GTT GTT GAC TCC GCG TGC-3`) was designed based on the partial pluranimaliumlysin (*pla*, EMBL_CDS: CBY79009.1) gene sequence of *A. pluranimalium* type strain 13483^T. Specificity of the PCR assay was evaluated using the *A. pluranimalium* type strain as a positive control and eight non-target strains (*A. haemolyticum* DSM 20595^T, *A. hippocoleae* DSM 15539^T, *A. phocae* DSM 10002^T, *Trueperella abortisuis* DSM 19515^T, *Trueperella bernardiae* DSM 9152^T, *Trueperella bialowiezense* DSM 17162^T, *Trueperella bonsai* DSM 17163^T, *Trueperella pyogenes* DSM 20630^T).

For amplification, mixtures (total volume 50 µl) containing GoTaq Green Master Mix (Promega, Madison, WI) with a final concentration of 1.5 mM $MgCl_2$, 200 µM dNTPs each and primers at 10 pmol concentration were prepared. Thermal cycling was carried out using an initial denaturation step of 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 58 °C for 30 sec and elongation at 72 °C for 30 sec. The amplification product was resolved on a 1 % agarose gel followed by ethidium bromide staining and examination under UV light. The PCR assay was successfully performed using either (10 ng) of extracted DNA (DNeasy® Blood & Tissue Kit, Qiagen, Switzerland) or boiled colony material.

With the primer pair PlaF/PlaR the *A. pluranimalium* type strain as well as isolate 704 target revealed a correctly sized amplification product (458 bp), whereas no amplification product was obtained for all non-target strains (100% specificity). The sequenced product of isolate 704 (JX144333) displayed 100% identity to the respective sequence of the type strain. The established PCR provides a convenient tool for the mastitis diagnostics to differentiate *A. pluranimalium* from *T. pyogenes*.

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4.6 Figures

Figure 1: Small, round, translucent to grey colonies of *A. pluranimalium* producing total hemolysis on blood agar.

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